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REVIEW

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Analytical techniques for multiplex analysis of protein biomarkers

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ABSTRACT

Introduction: The importance of biomarkers for pharmaceutical drug development and clinical diagnostics is more significant than ever in the current shift toward personalized medicine. Biomarkers have taken a central position either as companion markers to support drug development and patient selection, or as indicators aiming to detect the earliest perturbations indicative of disease, minimizing therapeutic intervention or even enabling disease reversal. Protein biomarkers are of particular interest given their central role in biochemical pathways. Hence, capabilities to analyze multiple protein biomarkers in one assay are highly interesting for biomedical research.

Areas covered: We here review multiple methods that are suitable for robust, high throughput, standardized, and affordable analysis of protein biomarkers in a multiplex format. We describe innovative developments in immunoassays, the vanguard of methods in clinical laboratories, and mass spectrometry, increasingly implemented for protein biomarker analysis. Moreover, emerging techniques are discussed with potentially improved protein capture, separation, and detection that will further boost multiplex analyses.

Expert commentary: The development of clinically applied multiplex protein biomarker assays is essential as multi-protein signatures provide more comprehensive information about biological systems than single biomarkers, leading to improved insights in mechanisms of disease, diagnostics, and the effect of personalized medicine.

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Article highlights

- High-throughput methods to validate proteins of choice by multiplex methods in a robust, time-efficient, and cost-effective manner are in place to further drive protein biomarker research and implementation in the clinic.
- Clinical immunoassays are established as a robust platform for multiplex protein analyses. Recent improvements in protein binding, detection, and miniaturisation have yielded robust assays to semi-quantitate a large number of preselected protein biomarkers in integrated assay panels. Particular attention has to be given to the characterization of protein binders as this drives assay specificity, selectivity, sensitivity, and throughput.
- Mass spectrometry has the unique capability to detect proteins and their post-translational modifications in an unbiased manner, reducing the necessity of selective protein binders. New developments in PRM, DIA, SWATH, FAIMS, and IMS will further improve the resolution and throughput of protein analytics. An elaborate understanding and solution of ion suppression will be found to increase the protein sensitivity.
- Alternative technologies in immunoassay based on optical label-free technologies will mature to a sensitive, multiplex, and label-free detection of biomarkers.
- The concomitant experimental development of nanotechnology-enabled resonator-based biosensors, site-directed bio-functionalization techniques, multi-spectral analytical approaches, and single-molecule protein sequencing will open promising perspectives for high-throughput protein clinical detection systems.

1. Introduction

Proteins are important mediators and actors in biology. Through their multiple functional roles as enzymes, cellular signaling components, neurotransmitters, cofactors or structural components, proteins affect the metabolic state and activity of cells, tissues, and organisms. Over the last century, proteins have been extensively studied, particularly their level of expression, modification, and interaction, as well as the dynamics involved. This understanding has been used to select proteins as potential drug targets and biomarkers to drive development of innovative therapeutic drugs. More recently, differences in protein biology associated with different pathologies, in some cases in combination with genetic variations, have been used to drive personalized medicine, whereby individually optimized therapies are based on these molecular changes [1].

Personalized medicine is from its conception driven by biomarkers that provide a comprehensive view of a specific human biological system. These biomarkers include different types of molecules (proteins, DNA, RNA, metabolites), cellular and tissue morphologies, and functional read-outs [2]. One of the first clinical demonstrators of biomarker-driven patient stratification toward higher clinical success was the combination of trastuzumab with the HER-2/neu immunohistochemistry assay [3], followed by other successes in oncology and beyond. As a consequence of increasing biological understanding, researchers have a rapidly growing need to measure multiple functional biomarkers, preferably in a simultaneous way. This review focuses on analytical methods that allow multiplex quantification of proteins and can potentially be used to quantify protein biomarkers in the clinic.

The overall workflow for multiplex protein analysis is in several aspects similar to singleplex protein analysis: 1) define the context of use, 2) select the proteins to be studied, 3) develop sample preparation methods that fit the measurement of the selected proteins, 4) develop analytical methods to measure the selected protein parameters, 5) develop data analysis methods to interpret the data in a biological context. However, in multiplex protein analysis inter-protein effects can complicate assay development and application. In addition, the need for stringent quality control of assay reagents to avoid research irreproducibility [4] is increased when multiple proteins are targeted in one assay. For that reason, researchers need to carefully select the analytical platform for multiplex protein tests.

Recently, researchers including us urged for the need to define best biomarker practice guidelines to bridge translational innovation gaps and improve the field of biomarker research and development [5,6]. We subsequently formed the pan-European COST CliniMARK consortium with academic and industrial biomarker scientists to define best biomarker practices. Among the items addressed, there was a strong focus on translational protein biomarker assays. In fact, despite the widespread knowledge of singleplex protein analyses, there is still limited information on the various methods and platforms available for clinical multiplex protein testing. Furthermore, regulatory guidelines for multiplex protein diagnostic tests are emerging but still have to find their way to research and health-care communities.

Here, we compare several techniques for multiplex analysis of proteins and include a pragmatic head-to-head comparison of multiplex analytical approaches. We focus on soluble (or solubilized) proteins using assay platforms with clinical potential. Immunoassay and mass spectrometry-MS methods are reviewed as the predominant platforms, and emerging methods with potential for further development into clinical application are also presented. In addition to analytical qualities, pre-analytical aspects are also essential in biomarker research. In this review, pre-analytical aspects are only considered in relation to a few specific analytical approaches. More general and comprehensive information about the importance of pre-analytical techniques is described elsewhere [7].

2. Sample selection and preparation

Validation of protein biomarkers requires the use of a robust assay to analyze hundreds, if not thousands of samples, standards, and calibrators. Since the final goal is the implementation of the test in the clinic, it has to be highly reproducible, capable of standardization, scalable for automation and high throughput, and implementable for users of diverse skills. Hence, moving into the validation phase is not only about confirming a good or excellent biomarker response in relation to disease, but also about demonstrating the potential to translate the procedures to open-access platforms and multi-user interfaces. Sample preparation should be simple, preferably comprising a few steps to minimize experimental variations and analytically validated. Appropriate internal and external controls should be included to enable quality assessment when comparing batch-to-batch analyses over time. With this in mind, we provide a guide to the preferable sample sources and preparation methods for biomarker validation studies (Figure 2, 3).

Selection of the sample type is critical. Whereas solid tissues are ideal for biomarker discovery since they are the source of biochemical alterations, they fall short in the validation stage. Solid tissues are inherently complex comprising multiple cell types, presence of

blood and can vary substantially from patient to patient. Physical (homogenization, cryo-pulverization, extrusion, and sonication), chemical (detergents, chaotropic salts), and/or enzymatic (matrix proteases) treatment steps are required to extract proteins from solid tissues creating a source of experimental variation. Hence, quantitative interpretation of protein expression in solid tissues is challenging and impractical for longitudinal studies. Newly emerging strategies to isolate and clonally grow specific primary cells as organoids to simulate tissue microenvironments is gaining traction in understanding the molecular processes of disease for individual patients and lends itself ideally for proteomics analysis [8]. Due to the relatively small size of these primary cells, similar extraction methods to those used for cell line cultures can be employed. As yet, organoids are not commonly used for biomarker validation but rather to select therapeutic agents.

Consequently, within the current regimen of acquiring clinical samples, which is aligned to immunoassay-based and mass spectrometry strategies, validation translates to more accessible sources such as biofluids. There are more than 30 biofluids, the most common being blood, cerebral spinal fluid (CSF), urine, saliva, tears, sweat, ear wax, interstitial fluid, and amniotic fluid. Biofluids or liquid biopsies require fewer preparation steps and are suitable for longitudinal collection from patients as part of continuous surveillance. They can also be a source of extracellular vesicles (e.g. exosomes) which contain molecular (protein, lipids, nucleic acids) information regarding specific diseases. Hence, liquid biopsies although surrogate for solid tissues, are more advantageous in the majority of validation studies.

Standardized procedures for collecting liquid biopsies are critical for immunoassay and proteomics analyses and data interpretation. Implementation of a collection protocol requires clear understanding between the clinician (e.g. consenting, patient reassurance), patient/volunteer (e.g. willingness to participate, fasting) and analyst (e.g. sample traceability, standard operating procedures). Where possible this should be within a controlled environment (e.g. storage for sample stability), even for readily collectible fluids such as saliva or urine. In addition, accurate, anonymized clinicopathological and patient personal data are essential to determine biomarker response related to the studied disease. A further consideration is the implementation of these procedures across different collection locations, whether these are hospitals, community centers, or at home. Even within the same type of settings, such as phlebotomy suites, understanding the variables (for example, different practitioners, different makes of storage vessel, transfer time from patient to storage location) are important variables, the tolerances of which need to be measurable and acceptable for a biomarker assay to viable. Ultimately, anonymized patient records, not only clinical data but also relevant personal data, are essential to enable interpretation of biomarker levels proportionate to disease specificity and not to unrelated confounding factors.

3. Immunoassays

3.1. ELISA

Enzyme-linked Immunosorbent Assay (ELISA) is a quantitative analytical method that measures antigen–antibody reaction via a color/light/fluorescent signal obtained by using an enzyme-linked conjugate and an enzyme substrate. This allows the measurement of the concentration of molecules in biological fluids or biological systems [9]. The technology was originally developed in 1971 as a follow-up on radioactivity-based assays [10]. It is a widely used method for research and clinical diagnostic purposes in different disciplines such as immunology, cancer, infectious diseases, and inflammation. It is a sensitive and specific test that produces rapid high-throughput results, with the advantage that it does not require complex equipment or radioactive labels.

ELISA assays can be found in different formats (Figure 1(a)). The most commonly used 'sandwich' ELISA format requires two antibodies specific for different epitopes of the antigen; a capture antibody and a detection antibody. The antigen-antibody complex can be visualized via color change, chemiluminescence, or fluorescent signals using an enzyme-linked conjugate (e.g. HRP – Horse Radish Peroxidase) and an appropriate substrate (e.g. TMB – 3,3′,5,5′-tetramethylbenzidine). In some cases, the visualization is based on a labeled secondary antibody being specifically bound to a primary detecting antibody [11]. The sandwich format offers higher sensitivity (pg/mL up to µg/mL) and specificity than direct and indirect ELISAs (using just one antibody) and enables the widespread use in preclinical and clinical laboratories. In addition, competitive ELISA [12] and in-cell ELISA (quantification of a target protein in cultured cells with a primary antibody; Figure 1(b)) [13] are techniques based on the same principle but with specific applications and throughput. The ELISPOT (Enzyme-linked Immunospot Assay) is a highly sensitive and quantitative sandwich immunoassay for measurement of proteins secreted by plated cells attached to supportive matrixes [14] (Figure 1(c)).

Although the ELISA is regarded as a singleplex assay format, the method can be made suitable for multiplex protein analysis. Monoclonal, polyclonal, or recombinant antibodies with a defined specificity, sensitivity, and stability can be combined in one test to allow binding of multiple proteins. In addition, the use of different fluorochromes to detect different antibodies enables multiple parallel read-outs, visualizing multiple proteins simultaneously [15]. These multiple readouts are combined in novel immunoassay formats in which different capture antibodies are immobilized on separate particles such as magnetic beads. Protein-specific fluorescence or streptavidin-labeled detection antibodies bind to the specific capture antibody-antigen complexes on a bead, after which each bead-antibody-antigen complex is analyzed by flow cytometry, yielding a multiplex analysis of up to 500 analytes per microwell (Figure 1(d)).

The ELISA principle can also be applied for single-cell protein secretome analyses using single-cell barcode chips capturing a panel of 32 pre-specified proteins from a single cell in each microchamber. The cells of interest may be stimulated and sorted before loading the cells on a chip. Chips are analyzed using Isoplex machinery, which contains automated cellular imaging and includes a complete ELISA workflow. The software provides information on the secreted proteins across different categories: homeostatic, proliferative, inflammatory, chemotactic, regulatory, and immune effector functions and provides a polyfunctionality strength index [16] (Table 1). Other commercially available methods for multiplex protein detection are reviewed below and summarized in Table 1.

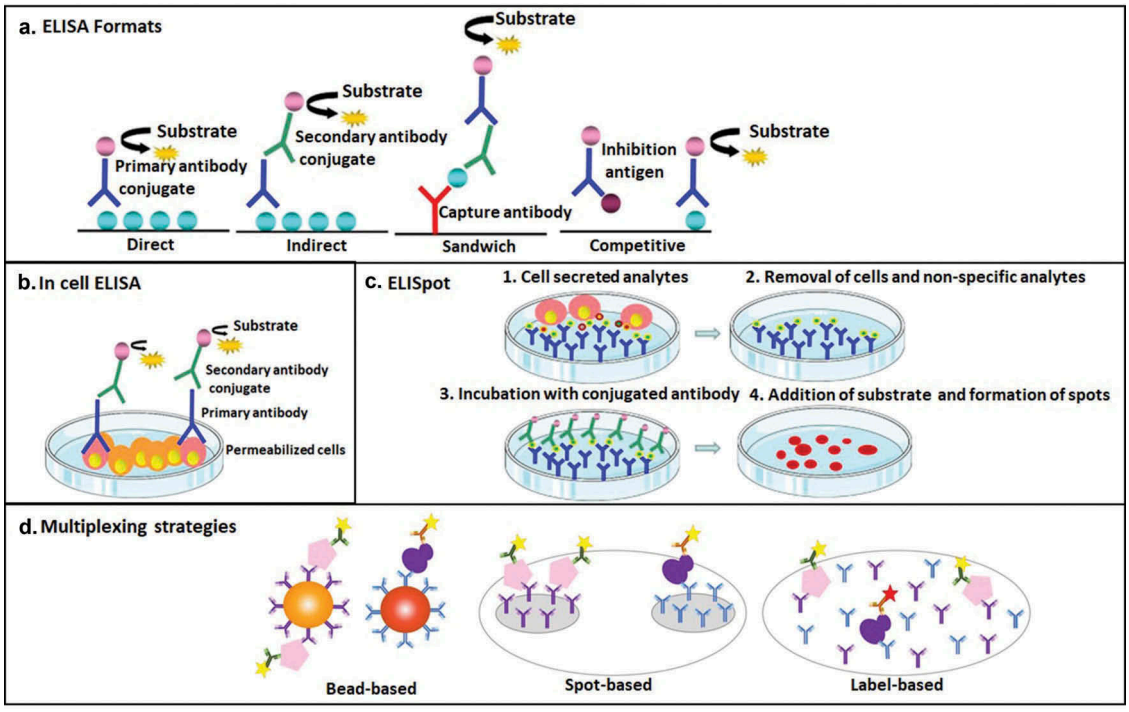


Figure 1. (a) – represents different ELISA formats and configurations. The resulting immune-complex is detected through an enzymatic reaction to produce a color, chemiluminescent, or fluorescent signal. (b) – represents 'in cell ELISA' for detection of intracellular analytes. (c) – represents ELISpot for detection of analytes secreted by cells. (d) – represents different strategies for immunoassay multiplexing. The bead-based approach relies on analyte-specific color-coding of fluorescent capture beads, while the label-based approach relies on the use of analyte-specific labels attached to the detection antibodies. The spot-based approach relies on physically separated analyte-specific spots.

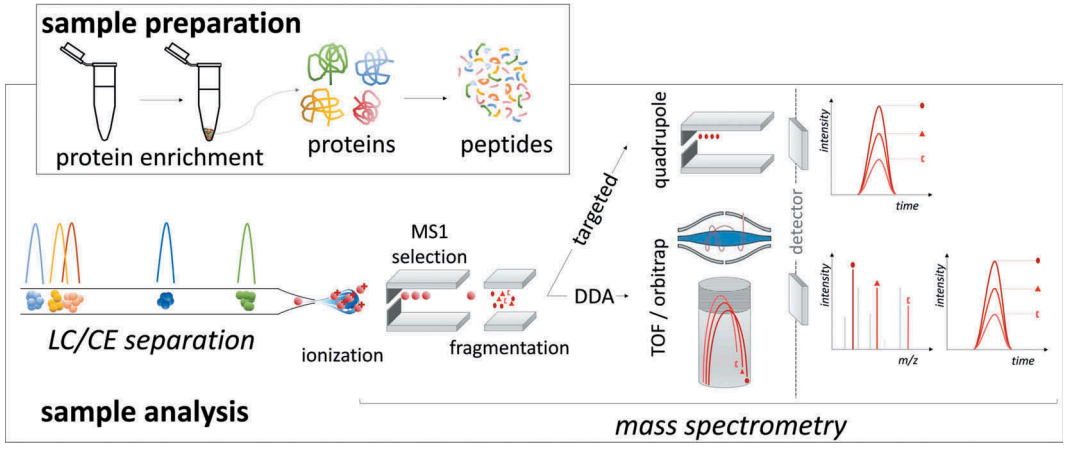


Figure 2. Representation of common MS-based proteomics approaches, i.e. targeted proteomics or data-dependent proteomics. In both bottom-up strategies, peptide detection is applied for protein identification or quantification. Generally, proteins are enriched, mainly by precipitation, and further denatured, reduced, alkylated, and digested chemically or enzymatically into peptides. After separating the peptides by liquid chromatography (LC) or capillary electrophoresis (CE), the ionization process converts the peptides into gas-phase ions, which enables the mass spectrometer to separate the ionized peptides based on their m/z ratios. Targeted proteomics (SRM/MRM/PRM) allows absolute quantification of selected proteins if stable isotope standards are included in the analysis. In classical label? free data-dependent acquisition (DDA) proteomics or 'shotgun' proteomics, peptide ions are specifically selected for fragmentation based on their detection intensity in precursor ion scans collecting MS/MS spectra for as many peptides as possible, which are identified against a proteomics database.

3.2. Chemiluminescence

Chemiluminescence detection is more sensitive than chromogenic technology to quantify multiple proteins. Spot-specific chemiluminescence detection by a charge-coupled device (CCD) camera is a plate-based multiplexing mini-array detection approach for high-throughput analysis of proteins. In this multiplex system, antibodies are spotted in a 96-well plate, and well-plate surfaces can be chemically modified to bind proteins. The samples are added to the wells and the target proteins captured by the antibody array. In the next step, biotinylated antibodies, specifically binding to the captured proteins, are added. Streptavidin-HRP conjugate and a chemiluminescent substrate are finally incorporated. A catalyzed oxidation of the



Figure 3. Decision matrix for the biomarker validation workflow using MS-based proteomics (font size defined by the number of papers found using keyword searches [biomarker + validation or verification + protein ± clinical + specific term, e.g. tissue or top-down] in Pubmed). The red-circled keyword is 'organoid,' a newly emerging 3D primary cell-based sample proving valuable in personalized biomarker validation, but representing only 0.036% of the 'sample type' papers at the current time.

substrate generates chemiluminescence at each spot with the captured targeted proteins, which is measured by imaging the entire plate using a compatible CCD camera technology. For the determination of each analyte concentration corresponding protein standards are applied [26,27]. Commercially available planar-based multiplex ELISA kits, requiring chemiluminescent detection for quantification of inflammatory markers in serum, plasma, urine, cell, and tissue lysate from different sources have also been produced [28]. Additionally, chemiluminescence-based single molecular analysis arrays are produced enabling performance of up to a 12-plex assay in a well [26]. (Table 1 and Figure 1).

Sensitivity of detection is further increased by electrochemiluminescence (ECL) in which the substances are brought to a higher excitation level *via* an electron transfer reaction. The electron transitions from a high excitation state to a much lower energy state are accompanied by light emission, and the emitted light can be detected, which enables ultra-sensitive analysis [29]. Despite that for many years this phenomenon was considered to be a part of fundamental research without an apparent practical application, ECL detection is now used to develop commercial devices to determine several clinically relevant analytes, including protein biomarkers and antibodies (Figure 4). An exhaustive overview of ECL bioapplications can be found in [30–32]. Commercial platforms, such as those of Mesoscale Discovery, exploit ECL as their method of detection [33]. Also, the possibilities of using ECL for point-of-care testing [34], microfluidics and paper-based technologies [35] or the combination of ECL with bipolar electrodes [36] have been evaluated at experimental level.

3.3. Lateral flow immunoassays

Lateral flow immunoassays (LFA) are successful systems in Point of care (POC) testing and used for both qualitative and quantitative monitoring in resource-limited or non-laboratory settings, for example in emergency settings [37]. LFA uses similar principles as ELISA, in which the capture antibody is immobilized to form a test line on a solid phase strip, e.g. nitrocellulose membrane, instead of a plastic well. The detection antibody is conjugated to a colorful particle or fluorescent label and this conjugate is allowed to freely react with the target analyte on the strip, when the sample is applied. As capillary action transfers the sample components through the test strip, the complex of the target analyte and detection antibody label-conjugate is halted at the test line by the capture antibody. The presence and/or concentration of the analyte in the sample can be determined by the intensity of the label at the test line [38,39]. Limited multiplexing of LFA can be achieved by incorporating multiple test lines for different analytes on the same strip or by creating an assembly of several strips around a common sample application pad. Several commercial LFAs have been used in the clinical world for biomarkers such as alpha-fetoprotein (AFP), carcinoma embryonic antigen (CEA), prostate-specific antigen (PSA), virus detection [40] and fecal occult blood (FOB). Both to automate and integrate LFAs with medical analyses, smartphone sensing has gained interest in the last decade [41,42] leading to the development of different POC systems for various diseases. Smartphone sensing technology measures colorimetric or fluorescence-based signals with an appropriate software or an app using colored molecules or fluorophores, respectively [43,44]. Thus, a term 'mobile health (mHealth)' which refers to the data sharing between patients and physicians emerges, including the use of websites and/or applications to specify and/or monitor a particular health condition [45]. The aim of mHealth is to achieve better personalized healthcare with providing an immediate resource for clinical decision by doctors and pharmacists [46].

3.4. DNA-based protein detection

Partly based on the immunoassay principle other platforms have emerged whereby protein binding by antibodies is combined with DNA-based detection using the advantages of signal

Table 1. Multiplex protein detection immunoassay techniques.

Multiplexing strategy	Supplier	Separation technology	Label attached to tracer antibody	Technology used for quantitation	Dynamic range (logs)	Detection limit[a]	Multiplexing	Reference
Spot-based	Meso Scale Discovery	antibodies as spots on wells	ruthenium chelate	spot-specific electrochemiluminescence detection by a CCD camera	3–4	0.6 pg/ml	10-plex	[33]
	Quanterix	antibodies as spots on wells	enzyme	spot-specific chemiluminescence detection by a CCD camera	2–4	0.04–0.4 pg/ml	10-18 -plex	[26]
	Raybiotech	Raybiotech	fluorophore	spot-specific fluorescence detection by a laser scanner	3–4	0.5 pg/ml	40-plex	[17]
Bead-based	Many suppliers	antibodies on paramagnetic beads	fluorophore	bead-specific fluorescence detection by a flow-cytometry type system or a chamber with CCD camera	2–4	~0.05–5 pg/ml	500-plex	[18]
	Abcam	antibodies on rod particles	fluorophore	particle-specific fluorescence detection by a flow-cytometry type system or from a chamber with CCD camera	5	0.12 pg/ml	70-plex	[19]
Label-based	PerkinElmer	antibody-coated wells	lanthanide chelate	time-resolved fluorescence detection from whole well	3–5	0.1 pg/ml	3-plex	[20,21]
	Assaypro	antibody-coated wells	fluorophore	fluorescence detection from whole well	2–3	8 pg/ml	4-plex	US Patent No. 9945847 (filed 2014, granted 2018)
	Merck	antibody-coated paramagnetic beads	fluorophore	eluted tracer antibodies detected individually by fluorescence	3–4	0.08 pg/ml	3-plex	[22]
	Quanterix	antibody-coated paramagnetic beads	fluorophore	digital (fraction of beads producing fluorescence) or analog (fluorescence intensity per bead) signal detection from an array of physically separated beads by CCD camera	3–4	0.006 pg/ml	10-plex	[23]
Special	Olink	no separation required	two tracer antibodies with oligonucleotides	qPCR (real-time fluorescence monitoring during PCR reaction)	3–5	0.9 pg/ml	92-plex	[47]
	SomaLogic	SOMAmer binders on beads	fluorophore	detection of eluted SOMAmers on a DNA array by CCD camera	8	~2 pg/ml	5000-plex	[24,25]
	IsoPlexis	antibodies as stripes in single cell microchambers	fluorophore	stripe-specific fluorescence detection by a specific reader	3–4	~10 pg/ml	32-plex	[16]

[a]Limit of detection for IL-6

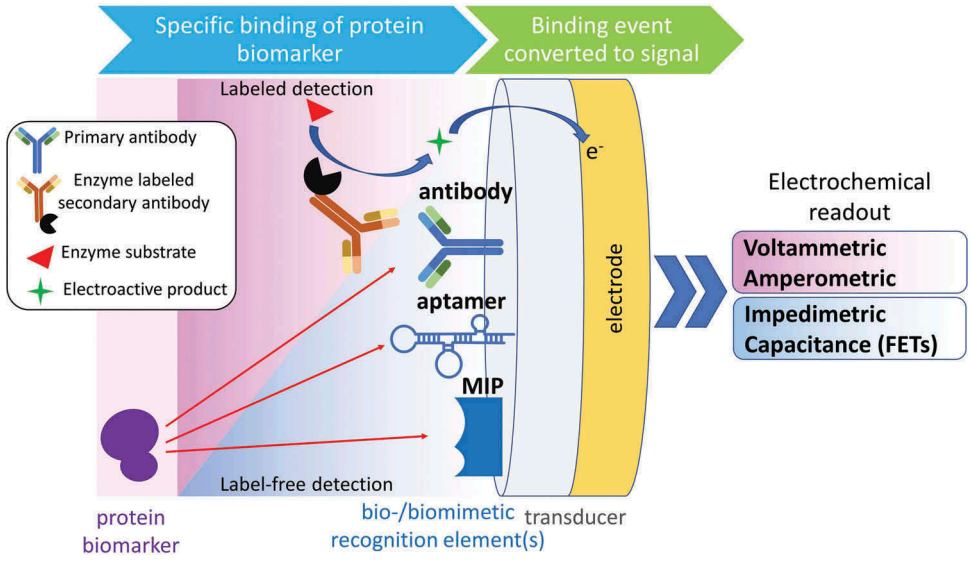


Figure 4. Labeled- and label-free strategies for electrochemical sensing of protein biomarkers. Various bio- or biomimetic recognition elements ensure the selective binding of the target protein, followed by the transduction of the binding event into a measurable electrochemical signal (MIP – molecularly imprinted polymer; FET – field-effect transistor).

amplification. In so-called proximity extension assays (PEA), a pair of antigen-specific antibodies are conjugated with complimentary oligonucleotides that produce a PCR target sequence when both antibodies bind to target protein in close proximity. After amplification, the DNA barcode is quantified by standard microfluidic qPCR using specialized equipment. The advantage of this approach is that it limits cross-reactivity as only matched DNA reporter pairs will be amplified. This allows high specificity and a higher degree of multiplexing in comparison to other multiplex assays (most of them limited to maximum of 10-plex assays). The PEA has been used to analyze a number of potential diagnostic or prognostic biomarkers in areas such as cancer and inflammation research [47]. Commercial PEAs facilitate multiplex assays enabling simultaneous detection of 92 analytes (in total >1100 pre-selected analytes are experimentally available) in only 1 µL of sample [47] (Table 1).

4. Mass spectrometry

Mass spectrometry is a powerful technique for detailed molecular characterization of biomolecules, including proteins, metabolites, lipids, and carbohydrates. Rather than just detection, mass spectrometry can reveal unique chemical fragments of such biomolecules, including post-translational protein modifications and specific metabolic isoforms of specific biochemical interactions [48,49].

Mass spectrometry (MS) is well suited for detection of proteins and proteoforms. Yet, because it also detects many other analytes, a thorough sample clean-up of clinical samples is required to remove contaminating salts and endogenous non-protein biological components. Furthermore, the huge dynamic range of proteins present in biological samples is a challenge for mass spectrometry analysis. For cultured cells and liquid biopsies such as urine and saliva, this difference is only 6–9 orders of magnitude whereas in plasma and tissue samples the dynamic range can be greater than 12, with serum albumin representing over 50% of the protein content. To overcome this, either abundant proteins are removed or target biomarkers enriched to enhance their detection [50]. In the former approach, samples (typically plasma or serum) are immunodepleted on columns that capture albumin, endogenous immunoglobulins, transferrin, fibrinogen, and apolipoproteins [51]. Proteins of interest do not bind to the columns and are recovered in the flow-through. In the latter case, protein enrichment may be carried out by subcellular fractionation using ultracentrifugation [52] or affinity chromatography with activity-based chemical probes to isolate specific protein classes based on structure and/or function (e.g. glycosylation, ubiquitination, kinases, phosphoproteins, metalloproteins) [53,54]. Immunoprecipitation, immunocapture, and capture by anti-peptide antibodies (SISCAPA) and mass spectrometric immunoassay (MSIA) are alternative methods dedicated to the enrichment of specific peptide or protein targets as these techniques provide the highest selectivity and sensitivity for low-abundant proteins [55,56] (Table 2). Precise and absolute quantification can be reached by the inclusion of stable isotope standards. Most ideally heavy labeled endogenous proteins are used for absolute and relative quantification of proteins of interest. However, for practical and financial reasons this is in many cases impossible, a second best solution is recombinant heavy labeled protein. This gives an identical concentration of the enzymatic generated peptides of the protein, although the folding and possible modifications present in the endogeneous protein are absent in the recombinant protein. A third and most commonly used option is the use of heavy labeled peptides. Because these peptides of one protein are chemically synthesized separately, differences can occur because of inaccuracies in determining the amount of peptide synthesized, different suppression of ions in the mass spectrometer cannot be corrected accurately for differences in behavior among the heavy labeled peptides and variances in recovery of the separate peptides from stock solutions can

Table 2. Multiplex protein detection mass spectrometric techniques.

Method	Supplier/Instrument	Separation technology	MS configuration	Clinical Application/Sample	Dynamic range (logs)	Detection limit *	Multiplexing	Reference
SRM	TSQ Quantiva	C_{18} RP chromatography	QQQ	FFPE breast cancer tissues	3	50–400 amol/ug	14-plex (5 proteins)	[57]
	Xevo-TQ XS, 5500 QTRAP	SISCAPA, C_{18} RP chromatography	QQQ	XLA, WAS, SCID, dried blood spots	3	0.7 to 1.4 fmol	5-plex (3 proteins)	[58]
	5500 and 6500 QTRAP	C_{18} RP chromatography	Q-iontrap	plasma, serum	NP	NP	182-plex (72 proteins), <20% CV	[59]
MRM	Xevo TQ-S	C_{18} Acquity	QQQ	plasma proteins	3–4	3–20 amol on column	15-plex (15 proteins)	[60]
	multiple platforms	C_{18} RP chromatography	QQQ	plasma	3–4	50–150 fmol/ml, 17 to 83 ng/ml	125-plex (34 proteins)	[61]
	Agilent 6490	C_{18} RP chromatography	QQQ	prostate cancer, urine	>5	NP	128-plex (51 proteins) single method, 213-plex (136 proteins)(2 methods)	[62]
	Xevo TQ-S	IPE or SCX, C_{18} RP chromatography	QQQ	serum, COPD, sRAGE	2	LLOQ 0.1 ng/ml	2-plex, 1 protein	[63,64]
	4000 QTRAP	SISCAPA, C_{18} RP chromatography	Q-iontrap	tamoxifen resistance prediction in ER-positive breast cancer	3–4	0.1 to 0.5 fmol/ml serum	8-plex (4 proteins)	[65]
	Quattro Micro	SISCAPA, C_{18} RP chromatography	QQQ	alpha tubulin acetylation, neurological disorders, rat brain	2–3	2.5 pmol per mg tissue	4 plex (1 protein)	[66]
	5500 QTRAP	SISCAPA, C_{18} nanoAcquity,	QQQ	oral cancer, saliva	2–4	0.4 to 1100 ng/mg protein	24-plex (24 proteins)	[67,68]
	TripleTOF 5600	C_{18} RP chromatography	QTRAP	cholangiocarcinoma	2–4	NP	3 plex (3 proteins)	[69]
	TripleTOF 5600	nanoLC400	QTRAP	rheumatoid arthritis		100 µg/ml	2-plex (2 proteins)	[70]
	LTQ Orbitrap Velos	C_{18} RP chromatography	Orbitrap	syphilis (*Treponema pallidum*), urine	1–2	10,000 organisms/ml	30-plex (11 proteins)	[71]
PRM	Q-Exactive	C_{18} RP nano-LC chromatography	Orbitrap	rheumatoid arthritis	NP	NP	14-plex (3 proteins)	[72]
	Q-Exactive Plus	antibodies (SISCAPA)	Orbitrap	cervical tumors, tissue	3	(LLOQ 0.07 to 1 ng/mg)	6-plex (6 proteins)	[73]
	Q-Exactive Plus	albumin and IgG depletion	Orbitrap	uterine aspirates	NP	NP	48-plex (26 proteins)	[74]
	Q-Exactive Plus	C_{18} RP nano-LC chromatography	Orbitrap	dried blood spot, ApoA-1 and ApoB	NP	140 and 83 µg/ml	9-plex (2 proteins)	[75]
	Oribtrap Fusion	C_{18} RP nano-LC chromatography	Orbitrap	cervical cancer, tissue	NP	0.2–1.9 fmol/ul	9-plex (4 proteins)	[76]
	Oribtrap Fusion	C_{18} RP nano-LC chromatography	Orbitrap	dementia, CSF	NP	0.11 to 8.63 ng/ml (LLOQ 0.37 to 27.65 ng/ml)	13-plex (7 proteins)	[77]
TMT 6-plex	Oribtrap Fusion	C_{18} RP nano-LC chromatography	Orbitrap	dementia, CSF (pleiotrophin)	NP	NP	singleplex	[78]
SWATH®	TripleTOF 6600	nanoLC	Quadrupole/TOF	HEK cell line	~4	4 to 8 µg protein digest	4500-5500 proteins, <20% CV	**
Signal intensity	micrOTOF MS	P/ACE MDQ capillary electrophoresis	ESI-TOF	urine (cancers, chronic kidney disease)	NP	peptidomics	CKD273	[79,80]
MS peak area	UltrafleXtreme	antibodies (SISCAPA)	TOF reflectron	cardiovascular disease, ApoA-1 and ApoB-100	1	NP	2-plex (2 proteins)	[81]
	Microflex LRF	antibodies (iMALDI)	TOF reflectron	breast cancer, colon cancer (AKT1 and AKT2)	1–2	LOQ 50 fmol/mg of protein	2-plex (2 proteins)	[82]

IPE (immunoprecipitation); COPD (chronic obstructive pulmonary disease); * (LOD unless otherwise stated); x-plex where x = the number of peptides analyzed; SCX (strong cation exchange); X-linked agammaglobulinemia (XLA); Wiskott–Aldrich Syndrome (WAS); CD3ζ-associated severe combined immunodeficiency (SCID), dried blood spots; NP – not provided; SISCAPA (stable isotope standards and capture by anti-peptide antibodies); sRAGE (soluble Receptor of Advanced Glycation End-products).** provided by the manufacturer.

occur. Despite the shortcomings of stable isotope labeled peptides, it is the most practical solution to perform the quantification of proteins with mass spectrometry. All these steps mentioned above are designed to assist identification of the protein of interest by proteomics-based mass spectrometry, but may increase sample preparation complexity and hence experimental variation.

Once the appropriate protein sample has been prepared, mass spectrometry can be employed to analyze intact proteins ('top-down' proteomics) or as peptides following proteolysis ('bottom-up' proteomics). In 'top down' proteomics, proteins are analyses as intact molecules rather than as smaller peptide fragments [83]. Analytical coverage of an intact protein still provides a strong challenge, although mass spectrometers capable of 'top-down' proteomics (MALDI MS with in-source decay, Orbitrap-MS^n, UHR-QToF MS, Fourier transform mass spectrometry-MS^n) are able to analyze an increasing number of intact proteins in one run [84]. Consequently, enrichment and affinity methods as described above play an essential role prior to intact protein analysis by MS. Although it circumvents the need for protease digesting proteins to peptides, 'top down' proteomics still has to be improved for broader applications in biomarker discovery and validation, and only limited examples of clinical applications are published [85] (Figure 3 and Table 2).

'Bottom-up' proteomics is the mainstay of biomarker research. However, sample processing methods in the translation from discovery to validation have a substantial impact on reproducibility, particularly when considering analysis of multiple experiments [86]. Whichever biological sources and extraction methods are used for validation studies, protein samples are likely to require clean-up (removal of detergents and other endogenous biomolecules) and necessitate concentration (e.g. urine) prior to proteolytic digestion [87]. Irrespective of the method used (protein precipitation with organic solvents/acids, dialysis, C_1 or C_4 reverse-phase cartridges, size exclusion chromatography and molecular weight cutoff spin columns), all additional steps risk lowering biomarker yield and increase potential for experimental variation. So far, most of these methods have not been universally standardized, validated, and automated for clinical use (Figures 2 and 3).

4.1. Mass spectrometry for biomarker validation – targeted proteomics (SRM, MRM, and PRM)

Targeted proteomics experiments are classically performed using triple quadrupole mass spectrometers in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) modes [88,89] (Table 2). SRM/MRM approaches have been widely applied for small molecule analysis and only recently emerged as a powerful analytical tool that can be used in 'bottom-up' proteomics, due to its wide dynamic range, relative and absolute quantification, and multiplexing capability. For each target protein, signature (or proteotypic) peptides are monitored using highly selective Liquid chromatography (LC)-MS/MS analysis. Particularly, the first and third quadrupoles (Q1 and Q3) of the instrument work as mass filters (typically with a window of ± 1 Da), successively selecting a series of signature peptide ions (Q1) and their corresponding fragments (Q3). Each precursor ion and its fragment ions are called transitions. The intermediate quadrupole (Q2) serves as a collision cell. Due to their multiplexing and scheduling capabilities (adjustment of transition monitoring at specific LC elution times), SRM experiments are especially adapted to large-scale evaluation studies of protein biomarker candidates [90]. Parallel reaction monitoring (PRM) is a similar acquisition mode specific to Orbitrap-type mass spectrometers. In this case peptide ions of interest are selected in a quadrupole in the same way as for triple quadrupole mass spectrometers, but then fragmentation occurs in a linear ion trap before rerouting all targeted product ions to the high-resolution Orbitrap simultaneously rather than sequentially. This enables higher sensitivity and more accurate quantification of the product ions.

In targeted proteomics experiments absolute quantification of candidate biomarkers is generally obtained using labeled peptide standards incorporating ^{15}N and/or ^{13}C isotopes [91]. These stable isotope labeled (SIL) (also referred as AQUA peptides) standards exhibit identical chemical and physical properties to the unlabeled target protein or its surrogate peptides, but they can be distinguished by their mass difference. According to the isotope dilution principle, SRM/MRM/PRM-based quantification can be performed at the peptide level by comparing transition signals from the isotope-labeled and the unlabeled version of a given proteotypic peptide. Different types of internal standards can be used, such as SIL peptides [92], winged SIL peptides [93], SIL peptide concatemers (QconCAT) [94], SIL protein fragments (QPrEST) [95] or full-length SIL proteins (PSAQ or absolute SILAC) [96,97]. Several comparative studies have shown that the quantification performance of targeted proteomics assays can be greatly influenced by the type and quality of the chosen standard [96,98,99]. In this context, the proteomics community has prepared specific recommendations to ensure the development of reliable and reproducible targeted proteomics assays, in a 'fit-for-purpose' approach [100,101]. Importantly, to promote the use of targeted proteomics assays, the Clinical Proteomic Tumor Analysis Consortium (CPTAC) has launched an Assay Portal to serve as an open-source repository of well-characterized targeted proteomic assays [102].

4.2. Data-dependent analysis (DDA) and data-independent analysis (DIA)

Historically most 'bottom-up' MS approaches use commercially available mass spectrometers (ion traps, Q-TOFs, IMS-TOF MS, Orbitraps, FT-MS, LC-MALDI MS/MS) for Data-Dependent Acquisition (DDA) compilation of proteomics data, particularly for discovery-based strategies. In the DDA strategy, as peptides pass through an LC column and enter the mass spectrometer, the instrument scans all ions but then chooses a subset of those, typically the most abundant ones (MS1 spectra), as precursor ions for further fragmentation usually by Collision-Induced Dissociation (CID). The fragmented ions obtained are then acquired to generate MS/MS data (MS2 spectra), which is used to identify peptides by reconstructing the peptide's amino acid sequence by database searching. Clinical applications of DDA workflows have proven to be robust and easy to implement in

the clinical setting [103,104]. Nonetheless, DDA strategy presents some disadvantages such as the speed of data acquisition, relative low reproducibility compared to immunoassay, absence of important information due to the low-abundant peptides that may not be available for fragmentation and lack of standardization of the quantitative strategy.

To overcome these limitations, a Data-Independent Acquisition (DIA) strategy was developed by Smith group [105] and later refined by the Aebersold's group in 2012 utilizing triple-TOF mass spectrometers, in which all theoretical spectra within sequential mass windows (known as SWATH-MS) are acquired [106–108]. A similar acquisition approach is possible on Orbitrap mass spectrometers, called WiSIM DIA [109] and on ion-mobility (IMS) mass spectrometers (MS^E) [110,111]. The idea behind DIA is to record all MS2 spectra from all precursor ions that fall in a specified mass-to-charge ratio (*m/z*) range, in a systematic and unbiased manner, leading to the entire set of peptide precursors of a sample. SWATH-MS technique is particularly innovative for discovery purposes due to its proposed data extraction strategy as it combines faster DIA with targeted data extraction. In each SWATH-MS measurement, data acquisition relies on consecutive and established m/z ranges of precursor ions that are isolated in order to be subjected to fragmentation. Through a rapid and recursive scan of these sequential *m/z* windows, the total precursor ion m/z range of previously digested proteins is covered and fragmentation spectra of precursor ions are obtained.

The DIA data obtained constitutes a complete digital map where ionized ions and specific peptides can be identified and/or quantified by applying targeted data extraction against a previously identified peptide list (also called library). These reference spectral libraries should contain all prior known information regarding peptide components of proteins in a study (e.g. retention time, precursor m/z or MS2 spectra) in order to be extracted from the DIA data. The peptide MS2 assay libraries can be generated locally through DDA analysis, obtained from community data repositories or even a combination of both. Thus, using an in-house generated library or combining it with a publicly accessible one, the overall information should ideally have high degree of protein coverage. The major repository of the peptide library assay [112], covering ~51% of human proteome (>10,000 proteins) is accessible to the scientific community for SWATH-MS analysis in human samples. Other public libraries have been made available by SWATH atlas (www.swathatlas.org). However, at this point, it seems conceptual that a locally generated in-house library is favored as it can be used for several SWATH-MS measurements, with the same LC conditions used to elute the unknown samples. This allows the retention times of eluted peptides to be within a similar range of those in the library. The quality and coverage of the library are of crucial importance for SWATH methodology performance. There are several detailed protocols [113,114] that have been successfully used to build a high-quality library for targeted analysis of SWATH-MS data, supported by DIA analysis software tools. An in-house library spectrum can be expanded at any time just with the addition of more information from DDA acquisition. To build more robust libraries, several sample processing strategies can be applied to reduce proteome complexity before LC-MS analysis, such as protein or peptide fractionation, PTMs or protein-specific enrichment, and depletion of most abundant proteins [114]. Thus, it must be taken into consideration that these procedures increase substantially the sample processing which may compromise analytical reproducibility.

DIA methodologies take advantage of high reproducibility and sensitivity of well-known target methods such as SRM, MRM, and PRM, with the increased proteome coverage normally seen in DDA analysis. In addition to its versatility and due to its capacity of fragmentation of the entire sample, the SWATH-MS technique is useful in characterization and/or quantification of low abundance proteins within a proteome, such as PTMs or other proteins that DDA (through shotgun analysis) approach cannot reach. Furthermore, SWATH-MS is a label-free methodology which is important for biomarker discovery but has yet to make an impact on clinical applications. Despite all of the advances achieved in the last years, DIA still has limitations in sensitivity as a tool for routine clinical diagnosis [107]. The definition of the best operating procedures, the standardization of protocols for sample preparation and acquisition between laboratories, as well as the need for appropriate and inexpensive internal standards will possibly globalize the method but the application of DIA strategies in clinic or large-scale studies is not reached yet at this stage. Currently, efforts have been made in order to achieve these goals and this will contribute to the increase of both databases repositories (spectral libraries and digital maps) (Table 2).

5. Alternative and other emerging technologies

5.1. Alternative protein binders

An impressive number of antibodies (monoclonal, polyclonal) have been produced and are commercially available for binding specific proteins in biomarker assays. However, through post-translational modification such as truncation, phosphorylation, acetylation, glycosylation, many proteoforms can be produced from a single gene product [115]. In the case where a higher specificity for a particular proteoform is needed in a biomarker assay, specific affinity matrices are required and can be too time-consuming, inefficient, or impossible to generate the right antibodies for each candidate biomarker to be validated. To that end, alternative binders have become available to replace or supplement antibodies. Firstly, affimers are small and stable recombinant proteins that are based on a stable protein scaffold, derived from the cysteine protease inhibitor family of cystatins. They contain two variable binding loops of nine amino acids each, which can be replaced with alternative randomized sequences to generate affimer libraries of ~10^{10} clones. These can be screened for affimers with specific properties such as high specificity and/or low dissociation rate [116]. Of interest, affimers are heat and pH-stable, expanding their application as protein affinity matrices in assays. Secondly, aptamers are small molecules that can be synthetically generated using combinatorial chemistry, thus

generating libraries that can be screened for protein-specific binding. Interestingly, aptamers are bound by proteins through a conformational binding pocket, resembling drug target and distinguishing from the binding of surface epitopes by antibodies and affimers. SomaLogic developed aptamer-based multiplex biomarker assays, measuring more than 1,300 protein analytes in a small volume of biological matrices in a single run. The detection technology uses Slow Off-rate Modified aptamers with dual nature for high protein affinity, with defined three-dimensional structure and unique nucleotide sequence which is recognizable by specific DNA hybridization probes [117,118]. Thirdly, less specific affinity matrices were developed to enrich specific protein classes based on their physical-chemical properties, such as lectins that have a higher affinity for glycosylated proteins [119]. However, in most cases, an additional high resolution of specific detection method is needed which could be provided by mass spectrometry.

5.2. Alternative protein separation

Capillary electrophoresis (CE) is a collective term representing a number of electrokinetic separation techniques performed in narrow bore capillaries or microchips. Capillary zone electrophoresis (CZE) is widely used for the separation of charged species based on differences in their charge density. CE offers an outstanding separation efficiency for peptides and small proteins, being complementary with liquid chromatographic (LC) separations, both in 'top-down' and 'bottom-up' proteomics. Interfacing CE to MS has matured into a robust clinical investigational tool in several disease areas [120], offering fast separations with good analytical sensitivities in protein biomarker analysis [121]. The most popular interface of CZE-MS coupling is via electrospray ionization (ESI). Both sheath-flow and sheath-less interface designs are employed. To circumvent significant sheath liquid-mediated sample dilution and the limited sample loading capacity of CE, tapered emitters operating in the nanospray regime not only support lower flow rates of the sheath liquid, but also contribute to better desolvation, enhanced sensitivity, and better salt tolerance [122]. Miniaturization to a single microchip (MCE) with the use of an electrophoretic step prior to biomarker detection may lead to an attractive clinical diagnostic tool [123].

5.3. Multi-omics analysis platforms

Optimally, to obtain an in-depth view of biological systems DNA, RNA, proteins and metabolites can be analyzed in one assay. Recently, electrochemical-based biosensors have been developed in which a specific biorecognition element (e.g. antibody, nucleotide strand, or aptamer) bind their biomarker target with high specificity and are combined with sensitive read-out modules [124]. Such biosensors represent an ongoing developing field for fast monitoring and assessment of clinically relevant biomarkers usually from biological fluids. Varying working principles have been put into practice, from platforms containing antibodies or aptamers immobilized on nano-materials for electrochemical biointerfaces [125] to magneto-immunosensors [126] and to formulate multiplexed nanoscale biosensors systems [127] (Figure 4). As a commercial platform, nanostring developed fluorescent molecular barcodes linked to a specific binder in combinations that are custom-made or pre-designed for particular biomarkers. Following binding to their target, molecular barcode probes are detected digitally through microscopic imaging, thus detecting and counting up to 800 hundred unique biomarkers. Of particular interest, by mixing the binders, different moieties of biomarkers can be detected in one sample, including DNA, RNA, and proteins [128].

5.4. Photonic and plasmonic resonating structure arrays

During the last decade a significant improvement of the analytical performances of POC detection devices was enabled through the development of photonic and plasmonic resonating structure arrays. In a generic way, this signal-to-noise amplification strategy relies on resonance phenomena responsible for a stronger optical energy confinement at specific slots on the substrate. One protein biosensor design focuses on label-free approach based on micro-resonators – ring resonator and crystal cavities arrays – and localized surface plasmon resonance (LSPR) on biofunctionalized metal nanoparticles or nanoantennas [129–133]. The other main signal enhancement strategy focuses on the use of a metal nanostructured substrate and near-infrared fluorophore labels in immunoassays [134,135]. The excitation field enhancement generated at the nanoscale gaps between the metal structures results in an increase of the optical transition rate and thus a stronger emission [136]. In comparison to more conventional LFA-based nitrocellulose, this approach provides a high signal-to-noise ratio, larger dynamic range – typically up to 6 orders of magnitude – and reduced fluorophore autofluorescence [137,138].

5.5. Miniaturized sensors

The current trend of miniaturization of immunosensors using micro and nanotechnology promises to increase the sensitivity and multiplexing for the analysis of proteins and other biomarkers, while providing cost-effective alternatives to established assays [139]. Nanostructured electrodes can provide limits of detection at sub-nanomolar concentrations by the use of redox labels with the advantage of using simple portable instrumentation [140–142]. Nanoparticles can be used to increase the original concentration of the analytes using magnetic nanoparticles [143], as well as to increase the transduced signal by optical (plasmonic or quantum dots) [144–146] or electrochemical means (by redox reactions or by changing the conductivity) [147]. However, usually these approaches make the assays more complex due to the use of labels. Other transduction methods based on nanotechnology are evolving to develop label-free biosensors using optical, electrochemical, and mass transduction. Micro cantilevers can be functionalized for the detection of very low concentrations of proteins (fM) [148]. Plasmonic immunosensors based on the localized surface plasmon resonance (LSPR) [146,149] and immunoassays based on potentiometric measurements with immuno-field effect transistors (immunoFETs) [150,151] are promising technologies for label-free sensing offering ultralow sensitivities (fM), cost-effectiveness and the possibility for ultra-high multiplexing

combining several sensors with the concept of micro-arrays (immobilization of molecular libraries) [152,153]. These characteristics of transducers like plasmonic biosensors or immunoFETs make them excellent candidates for early detection of cancer-biomarkers and POC diagnostics [154–156]. However, most of the nanotechnology-based devices are still at the stage of 'proof of concept.' While some of them have passed the threshold to be commercialized for applications related to DNA, most of them in combination with PCR amplification [157], protein assays still have issues to compete in sensitivity and selectivity with respect to well-stablished methods such as ELISA [158].

5.6. Protein sequencing

The strong improvements of immunoassays, mass spectrometry, and other targeted platforms to analyze proteins in multiplex mode, as discussed above, can still be further improved. In that sense, we may be able to learn from the Next Generation Sequencing field where exponential developments have enabled parallel analysis of single DNA and RNA molecules in multiplex fashion. In particular, parallel single-molecule detection modules are developed for proteins as a nucleotide sequencing equivalent based on fluorescence, tunneling currents, and nanopores [159–161]. Although still early days, such high-throughput single-molecule protein sequencing analyses would revolutionize the proteomics field, providing comprehensive overviews of proteomes, targeted detection of high and low-abundant proteins, and the realization of single-cell proteomics.

6. Expert commentary

6.1. Validation of a method toward application

The validation of candidate protein biomarkers is an imperative component for application in pharmaceutical drug development and clinical sample analysis. Fast, sensitive, and reliable technologies are needed to execute this essential step, including selection of biomarkers for further development to robust assays. The multiplex approach, particularly in parallel read outs is required to reach large numbers of samples for validation in a high-throughput manner.

The sandwich-format immunoassay has been developed into a standardized, robust, and reproducible method and several multiplex versions of the method have emerged. Being a crucial component, the quality of antibodies (or alternative binders) needs to be highly specific and selective, thus being able to bind the target protein with minimal-to-no cross-reactivity and interaction with other proteins. Overall, this part of the process requires more attention in the validation steps. There are web-based resources, such as the Human Protein Atlas or Antibodypedia, that provide researchers with summarized information of available antibodies and their validation status. However, for antibodies and any binders developed for protein quantification it is highly recommended to characterize and confirm binding and cross-reactivity in the specific setting of the aimed method. For this purpose thorough analytical validation of the immunoassay should be performed preferably according to widely acknowledged guidelines such as those of the Clinical Laboratory Standards Institute (CLSI). For monoclonal antibodies, sequence information and epitope mapping information is helpful for standardization [162,163]. Immunoassay technologies show the possibilities for multiplexing with various read out systems. The limiting factor is the accessibility and quality level of the antibodies used. Alternative binders such as affimers or aptamers are expanding the possibilities to specifically bind proteins, by different interaction mechanisms and under conditions with diverse stringency.

In parallel, high-resolution mass spectrometry has inherent possibility to multiplex many proteins in one run without the use of specific binders. It has the potential to measure proteins at the sub nanogram/mL biofluid or sub ng protein/gram of tissue if appropriate sample preparation is applied. Although mass spectrometry techniques show very good reproducibility, the process is still time-consuming because of the serial approach of protein (or peptide) separation and detection. The less-than impressive sensitivity is caused mostly by ion suppression of peptides that elute simultaneously with the peptides of interest. So inherently low levels of detection can be reached although at the cost of lower throughput. Therefore, breakthroughs in the more efficient loading of ions in the mass spectrometer will increase the sensitivity. Developments such as IMS, FAIMS separation technology integrated online to the mass spectrometer and further improvements in DIA are experimental tools to reach the clinic. Of particular interest to biomarker scientists, mass spectrometry is particularly suited for analysis of post-translational modifications of proteins. These can strongly influence the stability, folding, interaction with other biomolecules, and thus activity of the protein. Mass spectrometry can provide such analysis relatively straightforward in an unbiased manner, in contrast to immunoassays where specific binders have to be developed. Novel experimental MS workflows such as IMS, that provide structural information on proteins, are expected to further increase the depth of protein characterization. Mass spectrometry does lead the way forward in providing the means for multiple, parallel analyte analyses, and in the context of multiple biomarkers the opportunity for increased diagnostic accuracy. The emergence of machine learning and artificial intelligence will further enhance the predictive capability of these complex multi-biomarker data sets to define disease susceptibility or onset [164,165].

We expect that alternative protein binders to supplement or replace antibodies will be strong drivers of clinical protein biomarker assays. Affimers, that are generated as recombinant proteins in stable scaffolds, with a high diversity, can be selected for a particular property as desired (specificity, selectivity, association/dissociation rates, stability) to a much better extent than antibodies. Also of interest, aptamers that mimic small molecules that are bound by particular enzymes will particularly detect a protein subpopulation that has drug target like properties, and as such complements the selection of antibodies based on surface binding affinity. Regarding protein biomarker detection, we view the emergence of molecular barcodes as particularly interesting as this enables the combination of different types of binders in one integrated analysis and the

possibility to achieve semi-quantitation of DNA, RNA, and proteins. Finally, we see very high potential if analytical protein platforms could adopt some principles of the exponential developments in next-generation sequencing, for instance by single-molecule sequencing through nanopores, as this would significantly improve throughput and clinical applications. Although examples for DNA are established, the application for proteins is still premature.

Concluding, several analytical platforms have been developed to analyze protein biomarkers in a multiplex manner, some of which have the potential to seriously impact the way we do future biomarker research and clinical implementation and will drive the impact of personalized medicine [166]. Mass spectrometers are for clinical use especially applied in metabolomics, drugs vitamins, antibiotic resistance. The clinical use of mass spectrometers for clinical use of proteins is near future. Especially changes in Medical Device Regulation within Europe will change and accelerate this process of quantifying proteins of interest in a clinical environment (https://ec.europa.eu/health/sites/health/files/docs/20200325_news_md_en.pdf).

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